

**REMARKS**

Claims 1, 2, 4-16, 18, 20, 22-25 and 27-34 are pending in this application. Claims 29-34 are withdrawn from consideration as being drawn to a non-elected invention. Claims 14-16, 18 and 20 were objected to under 37 C.F.R. 1.75(c). Claims 1, 2, 4-16, 18, 20, 22-25, 27 and 28 were variously rejected under 35 U.S.C. § 112, first paragraph. Claims 1, 2, 4-16, 18, 20, 22-25, 27 and 28 were variously rejected under 35 U.S.C. § 112, second paragraph.

By this amendment, claims 12, 14-16, 18, 20, 25, and 29-34 have been canceled, claims 1, 5, 6, 8, 11, 13, 22, 23, and 24 have been amended without prejudice or disclaimer of any previously claimed subject matter. Support for the amendments can be found, *inter alia*, throughout the specification.

The amendments are made solely to promote prosecution without prejudice or disclaimer of any previously claimed subject matter. With respect to all amendments and cancelled claims, Applicant has not dedicated or abandoned any unclaimed subject matter and moreover have not acquiesced to any rejections and/or objections made by the Patent Office. Applicant expressly reserves the right to pursue prosecution of any presently excluded subject matter or claim embodiments in one or more future continuation and/or divisional application(s).

Applicant has carefully considered the points raised in the Office Action and believe that the Examiner's concerns have been addressed as described herein, thereby placing this case into condition for allowance.

**Specification**

Submitted herewith is a substitute specification excluding the claims as required according to 37 C.F.R. §1.125(a). Also submitted is a marked up copy of the specification is submitted with markings showing all the changes relative to the immediate prior version of the specification of record. Accordingly, changes to the specification made in the Amendment and

Response filed June 14, 2004 and the Preliminary Amendment filed September 5, 2002 are not marked in the substitute specification. *The substitute specification does not include new matter.*

#### Objection to Claims

Claims 14-16, 18 and 20 were objected to under 37 C.F.R. 1.75(c) as allegedly being of improper dependent form for failing to further limit the subject matter of a previous claim. Claims 14-16, 18 and 20 have been canceled. Accordingly, this objection may be properly withdrawn.

#### Rejection Under 35 U.S.C. § 112, Second Paragraph

Claims 1, 2, 4-16, 18, 20, 22-25, 27 and 28 were rejected under 35 U.S.C. 112, second paragraph as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Although Applicant believes that the claims were sufficiently definite when considered in view of the specification and the understanding of those of skill in the art, Applicant has attempted to respond to the concerns of the Examiner in order to enhance clarity and to facilitate disposition of the present case.

The claimed invention is directed to a method for generating a Drosophila clipped FRT (cFRT) chromosome incapable of reacting with a P transposase but capable of reacting with a yeast site specific flippase recombinase (FLP). The claimed invention is also directed to a method for generating a Drosophila clipped FRT2L2R (cFRT2L2R) chromosome incapable of reacting with a P transposase but capable of reacting with a yeast site specific FLP.

In claim 1, as amended herein, when a Drosophila FRT chromosome containing a P[FRT] insertion with a selection marker gene is treated with P transposase, transposition of the FRT chromosome occurs and FRT chromosomes are generated having many different sequences due to the local and random transpositions. These are referred to as the first FRT chromosome in the claimed method. The first FRT chromosomes are screened for the presence of the selection marker gene and the second FRT chromosome is obtained as a first FRT chromosome lacking the

selection marker gene. Therefore, the second FRT chromosome is not be able to interact with P transposase due to lacking the element of the P[FRT] insertion.

The second FRT chromosome is then analyzed for recombination capability and homozygous viability to test its suitability for genetic experiments. The recombination capability represents the functional activity of P[FRT] insertion and the homozygous location in comparison with original P[FRT] insertion. The homozygous viability assay represents the genetic information which is not detectably changed after its exposure to the P transposase in a *Drosophila* incubation system, as described in paragraph 0060 of the specification.

When the second FRT chromosome incapable of reacting with a P transposase is identified, its capability of reacting with a yeast site-specific flippase recombinase (FLP) in the second FRT chromosome is examined. This procedure is described in step (c2) and those with high recombination accessibility are selected as the third FRT chromosome. In the last step (d), the *Drosophila* clipped FRT (cFRT) chromosome is obtained after the third FRT chromosome is exposed to the P transposase and the steps (c1) and (c2) are repeated.

For the method of claim 22, a *Drosophila* double FRT chromosome containing a first P[FRT] insertion with a first selection marker gene on one arm of the chromosome and a second P[FRT] insertion with a second selection marker gene on the other chromosome arm is used. The *Drosophila* double FRT chromosome is treated with P transposase and the resulting first FRT chromosomes are screened for the presence of the selection marker genes and the second FRT chromosome is obtained as a first FRT chromosome lacking the selection marker genes. Therefore, the second FRT chromosome is not be able to interact with P transposase due to lacking the element of the P[FRT] insertion. The second FRT chromosome is then analyzed for recombination capability and homozygous viability. When the second FRT chromosome incapable of reacting with a P transposase is identified, the capability of reacting with a yeast site-specific flippase recombinase (FLP) in the second FRT chromosome is examined. This procedure is described in step (c2) and those with high recombination accessibility are selected as the third FRT chromosome.

In the last step (d), the cFRT2L2R chromosome is obtained after the third FRT chromosome is exposed to the P transposase and the steps (c1) and (c2) are repeated.

The Examiner has indicated that terms such as screened products, candidate products, desired products, sensitivity, insensitivity, and immobility lead to this rejection. Applicant respectfully submits that these words are widely used in the bioscience field. Despite this, the claims have herein been amended in order to enhance clarity of the claims. Specifically, after the FRT chromosome is treated by P transposase, it is designated as “the first FRT chromosome.” The screened products and candidate products have been designated as “the second FRT chromosome” and “the third FRT chromosome,” respectively. The second FRT chromosome is screened for and identified by the condition of the *Drosophila* not showing the properties of the selection marker gene in the P[FRT] insertion. And the third FRT chromosome is selected from the second FRT chromosome after it is analyzed by the recombination capability and homozygous viability in the *Drosophila* incubation system. A preferred embodiment is shown in the paragraph 0056 of the specification. The phrase “the desired products” refers to the subject of the present invention, i.e. a *Drosophila* clipped FRT (cFRT) chromosome which is incapable of reacting with a P transposase but is capable of reacting with a yeast site-specific flippase recombinase (FLP).

The Examiner states that the term “homozygous viability” “is indefinite because it is unclear how to screen the viability of a non-living biological molecule (i.e., the cFRT). Furthermore, it is unclear how this homozygous viability could in anyway represent a genetic background.” Office Action mailed January 13, 2004, page 9.

Applicant respectfully submits that the term “homozygous viability” is a well-known technology to examine the lethal genes in the field of *Drosophila*-related research. For example, a reference published in *Genetics* 53: 27-46 in January, 1966 demonstrates the technology and is entitled “Homozygous viability and fertility loads in *Drosophila melanogaster*”. At page 27 of the reference, paragraph 2, it states “[t]he homozygous viability load may be separated into two components, that due to lethal genes and that due to detrimental genes, which lead to a reduced level

of survival to adulthood.” Accordingly, homozygous viability is a characteristic well-known in the art.

As described in the reference, the information of the homozygous viability is analyzed by the character of *Drosophila*. Accordingly, the homozygous viability of the FRT chromosome is detected by a living fly, and not just screened via a non-living biological molecule. Furthermore, the homozygous viability does represent the genetic information of *Drosophila* according to the reference description.

Claim 11 was rejected for the recitation of the term “DNA configuration.” “DNA configuration” concerns the particular nucleic acid sequence, secondary structure, three dimensional structure, condensation level, etc., and the technical feature of claim 11 is to monitor the DNA sequence of the *Drosophila* cFRT chromosome. Accordingly, claim 11 is amended to recite “...monitoring a DNA sequence of said cFRT chromosome...”.

With regard to the page 8 of Final Office Action concerning “how a mutagen or an X-ray can cause the physical movement of a chromosome”, when the chromosome is exposed in the environment of the mutagen or the X-ray, the mutagen or X-ray would induce many kinds of the mutations in the chromosome such as, for example, a point mutation, a sequence mutation, a missing sequence of chromosome, or one part of sequence moved into another chromosome. Applicant respectfully submits that the skilled artisan in the field of bioscience would easily understand the description of the mutation of the mutagen or X-ray.

In view of the foregoing, Applicant respectfully requests reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, second paragraph.

#### Rejection Under 35 U.S.C. § 112, First Paragraph

Claims 1, 2, 4-16, 18, 20, 22-25, 27 and 28 were rejected under 35 U.S.C. 112, first paragraph as allegedly failing to comply with the enablement requirement. The pending claims

have herein been amended to address the Examiners concerns under 35 U.S.C. §112, second paragraph. Applicant respectfully traverses this rejection under 35 U.S.C. §112, first paragraph as it may be applied to the amended claims.

The Examiner states that “the enablement rejection is set forth because of the overall indefiniteness of the method steps.” Final Office Action, page 5. The invention according to the amended claims is described above in the section addressing the rejections under 35 U.S.C. §112, second paragraph. Applicant respectfully submit that the claims clearly set forth the method steps in a definite manner.

Applicant respectfully submits that the specification provides all the information required for one of skill in the art to make and use the invention as claimed without undue experimentation. See, for example, paragraphs [0058]-[0066] and [0083]-[0092]. For a *prima facie* case on non-enablement, the burden is on the Office to demonstrate that there is a reasonable basis to question the presumptively sufficient disclosure made by applicant. See, for example, *In re Wright*, 27 USPQ2d 1510 (Fed. Cir. 1993). Applicant respectfully submits that a *prima facie* case for lack of enablement has not been established.

Applicant respectfully submits that the pending claims are in compliance with the enablement requirements. Accordingly, Applicant respectfully requests reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, first paragraph.

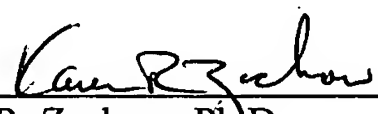
**CONCLUSION**

Applicant believes that all issues raised in the Office Action have been properly addressed in this response. Accordingly, reconsideration and allowance of the pending claims is respectfully requested. If the Examiner feels that a telephone interview would serve to facilitate resolution of any outstanding issues, the Examiner is encouraged to contact Applicant's representative at the telephone number below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 5298720000100. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

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Respectfully submitted,

By   
Karen R. Zachow, Ph.D.  
Registration No.: 46,332  
MORRISON & FOERSTER LLP  
3811 Valley Centre Drive, Suite 500  
San Diego, California 92130  
(858) 720-5191



***DROSOPHILA* CLIPPED *FRT* (cFRT) CHROMOSOME  
INSENSITIVE TO *P* TRANSPOSASE, GENERATING  
METHOD THEREOF, AND APPLICATION THEREOF**

FIELD OF THE INVENTION

[0001] The present invention is related to a *Drosophila* clipped *FRT* (cFRT) chromosome, and more particularly, to a *Drosophila* clipped *FRT* (cFRT) chromosome insensitive to *P* transposase but remaining ~~functional~~ sensitive to yeast site-specific flippase recombinase (FLP), the generating method thereof, and the application thereof.

BACKGROUND OF THE INVENTION

[0002] The human diseases, the screening of the pathogenic genes and the functional research thereof are always important research fields. ~~However, due to the systematic limitation, the possible living mechanisms are understood mostly through animal models which contain manipulated genes.~~ For hundreds of years, *Drosophila Melanogaster* have been used as the basic source material for teaching and genetics study. In the last few decades, since the molecular biology method was brought into this field, the research of *Drosophila* molecular developmental genetics has entered into a new stage. ~~[[The]]~~ Many biological mechanism mechanisms of *Drosophila* is are evolutionally conservative conserved in *Drosophila*. Therefore, from *Drosophila* study, people have come to understand that many human disease genes and cancer-related genes are derived from ~~[[the]]~~ gene mutation mutations which ~~controls~~ the are involved in developmental mechanism mechanisms. For example, the initiative mechanism of colon cancer and its complex working ~~process~~ processes are understood by studying the related genes of Wingless pathway which drives the development of abdominal segment formation in *Drosophila* embryo. Therefore, ~~the main future studying direction of the present invention is doing the~~ provides techniques for searching and studying of the specific functional genes in *Drosophila* genome ~~based on the technique of the present invention.~~ The *Drosophila* model will then be the base of searching the homologous genes of the human disease and pathogenic genes for the further study of human genetic mechanism.



[0003] In the present time, as the structural genomic projects in model organisms are completed, how to decipher a large amount of raw DNA sequences data in understanding gene function *in vivo* will be one of the major tasks for biology-related researchers. Different genomic strategies for defining and dissecting developmental and physiological pathways have been approached. The summit of these approaches is the systematic genomic screening of a specific functional trait using DNA tags, such as P-transposon or retrovirus, as mutagenesis agents. The designed transposon can be used as a mobile element to rapidly obtain cellular DNA sequence nearby the genetic mutation. The cellular DNA sequences can be obtained from the P-transposon-induced mutated genes loci by the direct use of inverse PCR (IPCR) or plasmid rescue methods. ~~By such operating way~~ Using such methods, the inefficient and labor-intensive drill of cloning sequences for obtaining the junction sequence between the host and the mobile element can be circumvented. While retrovirus was used to mutate leukemia-causing genes in mouse (Li et al., 1999, Nature Genetics 23, 348), several hundred integration sites were cloned and characterized followed by high-throughput sequencing, data analysis and refined genetic mapping. Similarly, a global transposon mutagenesis in Mycoplasma allowed the question of the number of essential genes in a minimal genome to be answered (Hutchison, et al., 1999, Science 286, 2165). Therefore, ~~by combining with~~ the combined use of emerging genomic tools, ~~such systems~~ in model organisms will indeed dramatically accelerate the pace of discovery in human disease-genes and cancer-related genes.

[0004] For the functional study of *Drosophila* genes, a conventional approach relies on the creation of mosaic animals whereby the genotype varies in a cell-specific or tissue-specific manner. Currently, various techniques utilize the yeast FLP-FRT recombination system introduced into *Drosophila* (Golic and Lindquist, 1989, Cell 59, 499) to promote chromosomal site-specific exchange. This system allows the efficient recovery of homozygous patches in an otherwise heterozygous animal and thus permits a phenotypic analysis of mutant tissues.

[0005] Different versions of the FLP-FRT (Flippase-Flippase Recombination Target sequence) system have been established for analyzing gene functions in either somatic or germline tissues. The direct mosaic productions in different somatic tissues have been established (Xu and Rubin, 1993, Development 117, 1223; Duffy et al., 1998, Development 125, 2263). In these methods, different tracing markers are used as the controls to monitor the presence of homozygous clones of genes to be studied. In addition, the FLP-DFS technique suitable for asking germline functions for loci residing in more than 95% of the genome has also

been systematically completed (Chou and Perrimon, 1992, Genetics 131, 643; Chou et al., 1993, Development, 119, 1359 and Chou and Perrimon, 1996, Genetics 144, 1673). The FLP-DFS technique uses the X-linked germline-dependent dominant female sterile mutation *ovo<sup>DI</sup>* as a selection marker for the detection of germline recombination events. Nevertheless, the FLP-FRT system is used to promote site-specific chromosomal exchange (Chou and Perrimon, 1992, Genetics 131, 643).

[0006] However, the major drawback for all of these FLP-FRT methods is that the mobile element, such as the P-transposon, can not be used directly as the mutagenesis agent to mutate the FRT chromosomes. While  $\Delta 2-3$  transposase is recognizing the *P* transposon insertion as the mobilization origin, it simultaneously recognizes and transposes the *P[FRT]* insertions used in the FLP-FRT system. Under such a situation, the genetic recombination cannot proceed due to the fact that the *P[FRT]* chromosomes are not homologous. For example, the mobilized *P[FRT]* will mostly create a non-homologous condition. However, the germline recombination of *P[FRT]-ovo<sup>DI</sup>* chromosome needs the existence of the homologous *P[FRT]* chromosome when *P[FRT]-ovo<sup>DI</sup>* chromosome is used for the FLP-DFS germline recombination. Therefore, the transposition of the *P[FRT]* insertion results in a non-homologous condition so that the germline recombination cannot proceed.

[0007] Presently, only EMS-based methods can be used for a full-scale genome-wide screening when using FRT chromosomes. Many interesting genes have been recovered. However, the goal to completely recover and to do molecular characterization of all interesting loci efficiently would be difficult if only EMS-based methods are used for mutagenesis. Because EMS produces mostly point mutations, it does not create any molecular tags on mutated genes for cloning manipulation. Consequently, the approach for identifying important genes is heavily impeded by the inefficient and labor-intensive traditional molecular cloning procedures.

[0008] Another alternative strategy to facilitate gene cloning is to use transposition system independent of the P transposon. For example, the Hobo element system can be used to cause the gene mutation. However, the problem of creating a chromosomal environment that completely avoids the P transposon system has not been possible in the *Drosophila* field. This kind of approach has never been described in the field while the versatile FLP-FRT system has been publicized since 1989 (Golic and Lindquist, 1989, Cell 59, 499).

[0009] Another way to overcome this is to individually recover the recombinant chromosome with an interested *P* insertion and the specific *P[FRT]*. This tedious and laborious

work has been done for a collection of 496 P element-induced mutations established by the Berkeley *Drosophila* Genome Project (Perrimon et al., 1996, Genetics 121, 333). By using the FLP-DFS technique, 496 independent zygotic lethal mutations identified by single P-element mutations were tediously recombined with FRT chromosomes in order to analyze their germ-line clone phenotypes (Perrimon et al., 1996, Genetics 121, 333). Similarly, the same approach has been conducted in at least 7 labs in Europe. They collected only 700 recombinant chromosomes within at least 6 years. The strategy to perform similar recombination experiment is confronted with not only the prerequisite to know the location of the new P insertion for a successful recombinant but also the limitation of reaching a saturation screening since the recombination suppression exists in certain chromosome regions. These recombinants are too few to reach the 24,300 lethal chromosomes in order to reach an 87% saturation screening for the functional description of *Drosophila* essential genes (Spradling, 1999, Genetics 153, 135).

[0010] In order to overcome the foresaid drawbacks, the present invention circumvents the above difficulties by constructing an advanced version of *P[FRT]* insertions on the *Drosophila* second chromosome, which allow the P-directed mutagenesis to be useful for quick chromosome-wide screening and fast molecular cloning for the various FLP-FRT methods. Molecular biology technique such as inversed PCR (polymerase chain reaction) and plasmid rescue methods can be used to recover flanking genomic DNA sequences and relevant molecular properties of the genes affected by the transposon. Based on the mutated phenotypes of either germline or somatic recombinant clones produced, the biological functions can be described for the genes mutated. The integrated description of the molecular nature and biological function of *Drosophila* genes can accelerate the understanding of the function of human gene homologues and be used as the basis for the application and development of gene-based medicines.

#### SUMMARY OF THE INVENTION

[0011] It is an object of the present invention to provide a method for generating a *Drosophila* clipped FRT (cFRT) chromosome insensitive to P transposase but remaining ~~functional~~ sensitive to yeast site-specific flippase recombinase (FLP).

[0012] It is another object of the present invention to provide a method for constructing an advanced version of the two *P[FRT]* insertions on the left and right arms of the *Drosophila* second chromosome, thereby forming the cFRT<sup>2L2R</sup> chromosome insensitive to P transposase.

[0013] It is another object of the present invention to provide a cFRT<sup>2L2R</sup> chromosome, which allows the P-directed mutagenesis ~~to achieve purposes of~~ for use in quick chromosome-wide screening and fast molecular cloning for the various FLP-FRT methods.

[0014] It is another object of the present invention to provide a cFRT<sup>2L2R</sup> chromosome, which can be directly mutagenized by the P transposon and instantly followed by genetic recombination after the recovery of mutations.

[0015] According to the present invention, a method for generating a *Drosophila* clipped FRT (cFRT) chromosome ~~insensitive to~~ incapable of reacting with a P transposase but remaining ~~functional to~~ capable of reacting with a yeast site-specific flippase recombinase (FLP), comprises steps of: (a) exposing a FRT chromosome to the P transposase ~~for occurring causing~~ a local and imprecise transposition, wherein the FRT chromosome contains a P[FRT] insertion with a selection marker gene, (b) screening the P[FRT] insertion ~~insensitive to~~ incapable of reacting with the P transposase to obtain screened products, (c) selecting candidate products from the screened products by further examinations, and (d) exposing the candidate products ~~[[by the]] to~~ to P transposase and selecting a desired product by the further examinations to obtain the *Drosophila* clipped FRT (cFRT) chromosome ~~is insensitive to~~ incapable of reacting with the P transposase but remaining ~~functional to~~ capable of reacting with yeast site-specific flippase recombinase.

[0016] In accordance with the present invention, the method further comprises a step (e) of examining the actual molecular nature of the clipped insertion by PCR (polymerase chain reaction).

[0017] Preferably, the step (c) further comprises steps of: (c1) examining the screened products for both recombination capability and homozygous viability, and (c2) examining recombination accessibility of FRT sequences contained in a clipped P[FRT] insertion by the presence of the FLP to obtain the candidate products.

[0018] Preferably, the recombination capability represents the functional activity of the clipped P[FRT] insertion and its homologous location relative to that of the original P[FRT] insertion.

[0019] Preferably, the homozygous viability represents a genetic background after the chromosome's exposure to the P transposase.

[0020] Preferably, the step (d) of exposing the candidate products ~~[[by]] to~~ to the P transposase and selecting the desired product by the further examinations is repeated at least twice.

[0021] Preferably, the *Drosophila* cFRT chromosome is an isogenized homozygous viable *Drosophila* second chromosome.

[0022] Preferably, the cFRT is formed due to a target sequence, recognized by the *P* transposase and responsible for a *P* transposase transposition, which is ~~damaged and alternated~~ altered into a type of incomplete target sequence, through one of a group consisting of: (1) ~~missing~~ loss of a P5' DNA sequence region, (2) ~~missing~~ loss of a P3' DNA sequence region, and (3) ~~missing~~ loss of DNA sequences other than those defined in item (1) and in item (2).

[0023] Preferably, the *Drosophila* cFRT chromosome ~~remains the functional~~ retains activity of the cFRT insertion for a site-specific recombination in the presence of the FLP.

[0024] Preferably, ~~an effectiveness of~~ the *Drosophila* cFRT chromosome is monitored by a FLP-FRT system and derived modification systems thereof.

[0025] Preferably, ~~an effectiveness of~~ the cFRT chromosome is monitored by molecular biology methods for the description of the cFRT DNA sequences ~~configuration~~.

[0026] Preferably, the *Drosophila* cFRT chromosome ~~remains to behave normally as a~~ retains normal wild type chromosome behavior, feasible for various genetic manipulations.

[0027] Preferably, a clipped *P[FRT]* insertion is ~~alternatively~~ moved to another chromosome from the *Drosophila* clipped *FRT* (cFRT) chromosome by treating the *Drosophila* cFRT chromosome with one of mutagens and X-ray.

[0028] Preferably, the *Drosophila* cFRT chromosome is ~~alternatively~~ used to establish a *Drosophila* cell line based on a genetic background of the *Drosophila* cFRT chromosome.

[0029] Preferably, the *Drosophila* cFRT chromosome is mutated to obtain gene mutations for further experiment.

[0030] Preferably, ~~[[a]]~~ molecular information of the gene mutations is recovered by retrieving flanking DNA sequences of a clipped *P[FRT]* insertion with a molecular biology method.

[0031] Preferably, the molecular biology method includes a plasmid rescue method, an inversed PCR method and a chromosomal walking method.

[0032] Preferably, the molecular information of the gene mutations can be recovered by a related bioinformatic manipulation.

[0033] Preferably, the related bioinformatic manipulation includes blasting databank, searching gene homologues of biological organisms, analyzing comparative genomics, and analyzing phylogenic distance and relationship.

[0034] Preferably, the functional description of the gene mutations are further analyzed based on the information obtained from the molecular biology method and the related bioinformatic manipulation by using a biological technique.

[0035] Preferably, the *Drosophila* cFRT chromosome is used to study the *Drosophila* genes located on the second chromosome and their corresponding gene homologues of other biological organisms including vertebrates, invertebrates, eukaryotes and prokaryotes.

[0036] According to another aspect, a method for generating a *Drosophila* clipped  $FRT^{2L2R}$  (cFRT<sup>2L2R</sup>) chromosome ~~insensitive to~~ incapable of reacting with a *P* transposase but remaining ~~functional to~~ capable of reacting with a yeast site-specific flippase recombinase (FLP), comprises steps of: (a) exposing a double-*FRT* chromosome to the *P* transposase ~~for occurring~~ causing a local and imprecise transposition, wherein the double-*FRT* chromosome contains a first *P*[*FRT*] insertion with a first selection marker gene on one arm thereof and a second *P*[*FRT*] insertion with a second selection marker gene on the other arm thereof, (b) screening respectively the first *P*[*FRT*] insertion and the second *P*[*FRT*] insertion ~~insensitive to~~ incapable of reacting with the *P* transposase to obtain screened products, (c) selecting candidate products from the screened products by further examinations, and (d) exposing the candidate products ~~[[by the]] to~~ to *P* transposase and selecting a desired product by the further examinations to obtain the *Drosophila* clipped  $FRT^{2L2R}$  (cFRT<sup>2L2R</sup>) chromosome ~~insensitive to~~ incapable of reacting with the *P* transposase but remaining ~~functional to~~ capable of reacting with yeast site-specific flippase recombinase.

[0037] In accordance with the present invention, the method further comprises a step (e) of examining the actual molecular nature of the clipped insertions by PCR.

[0038] Preferably, the step (b) further comprises steps of: (b1) screening the first *P*[*FRT*] insertion insensitive to the *P* transposase ~~subject to an~~ based on immobility of the first selection marker gene, and (b2) screening the second *P*[*FRT*] insertion insensitive to the *P* transposase from the screened products of step (b1) ~~subject to an~~ based on immobility of the second selection marker gene.

[0039] Preferably, the step (b) further comprises steps of: (b1') screening the second *P*[*FRT*] insertion insensitive to the *P* transposase ~~subject to an~~ based on immobility of the second selection marker gene, and (b2') screening said first *P*[*FRT*] insertion insensitive to said *P* transposase from screened products of step (b1') ~~subject to an~~ based on immobility of the first selection marker gene.

[0040] Preferably, the step (c) further comprises steps of: (c1) examining the screened products for both recombination capability and homozygous viability, and (c2) examining recombination accessibility of FRT sequences contained in the *P[FRT]* insertion by the presence of the FLP to obtain the candidate products.

[0041] Preferably, the first selection marker is different from the second selection marker.

[0042] Preferably, the *Drosophila* clipped  $FRT^{2L2R}$  chromosome is alternatively generated from two *Drosophila* clipped FRT (cFRT) chromosomes ( $cFRT^{2L}$  and  $cFRT^{2R}$  chromosomes) by a genetic recombination method.

[0043] According to another aspect, a *Drosophila* clipped FRT (cFRT) chromosome, wherein the chromosome is ~~insensitive to~~ incapable of reacting with a *P* transposase but remains ~~functional to~~ capable of reacting with a yeast site-specific flippase recombinase (FLP), comprises: a *Drosophila* second chromosome main body, and a clipped *P[FRT]* (cFRT) insertion immobilized by the *P* transposase.

[0044] In accordance with the present invention, the cFRT is formed due to a target sequence, recognized by the *P* transposase and responsible for a *P* transposase transposition, which is ~~damaged and alternated~~ altered into a type of incomplete target sequence, through one of a group consisting of: (1) ~~missing~~ loss of a P5' DNA sequence region, (2) ~~missing~~ loss of a P3' DNA sequence region, and (3) ~~missing~~ loss of DNA sequences other than those defined in item (1) and in item (2).

[0045] According to another aspect, a *Drosophila* clipped  $FRT^{2L2R}$  ( $cFRT^{2L2R}$ ) chromosome, wherein the chromosome is ~~insensitive to~~ incapable of reacting with a *P* transposase but remains ~~functional to~~ capable of reacting with a yeast site-specific flippase recombinase (FLP), comprises: a *Drosophila* second chromosome main body, and a clipped *P[FRT]* (cFRT) insertion on a right arm ( $cFRT^{2R}$ ) of the *Drosophila* second chromosome and a clipped *P[FRT]* (cFRT) insertion on a left arm ( $cFRT^{2L}$ ) of the *Drosophila* second chromosome, wherein both the  $cFRT^{2R}$  and the  $cFRT^{2L}$  are immobilized by the *P* transposase.

[0046] In accordance with the present invention, the *P[FRT]* insertions on a left arm is inserted into the 3' end of the base T at 240696 bp of the AE003781 clone with the P3' end facing the centromere before being clipped, and said *P[FRT]* insertion a right arm is inserted into the 3' end of the base T at 11497 bp of the AE003789 clone with the P5' end pointing toward the telomere before being clipped.



[0047] Preferably, the cFRT<sup>2L</sup> is an imprecise excision caused by a removal of P5' region and most ~~[[part]]~~ of a selection marker gene thereon, wherein a fragment from bases 26 to around 2070 of FBtp0000348 locus is deleted.

[0048] Preferably, the cFRT<sup>2R</sup> is an imprecise excision caused by ~~[[a]]~~ removal of most of the P5' region and one of the FRT DNA repeats, wherein a fragment from bases 10 to 2821 of FBtp0000268 locus is deleted.

[0049] The foregoing and other features and advantages of the present invention will be more clearly understood through the following descriptions with reference to the drawings, wherein:

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0050] Fig. 1 (SEQ ID NO:34) is a diagram showing the position of the *P[FRT]* insertion on the left arm of the FRT chromosome before being clipped according to a preferred embodiment of the present invention;

[0051] Fig. 2 is a diagram showing the *P[FRT]* insertion on the left arm of the *FRT* chromosome before being clipped according to a preferred embodiment of the present invention;

[0052] Fig. 3 is a diagram showing the *P[FRT]* insertion on the left arm of the *FRT* chromosome after being clipped according to a preferred embodiment of the present invention;

[0053] Fig. 4 is a diagram showing the position of the *P[FRT]* insertion on the right arm of the *FRT* chromosome before being clipped according to a preferred embodiment of the present invention;

[0054] Fig. 5 (SEQ ID NO:35) is a diagram showing the *P[FRT]* insertion on the right arm of the *FRT* chromosome before being clipped according to a preferred embodiment of the present invention; and

[0055] Fig. 6 is a diagram showing the *P[FRT]* insertion on the right arm of the *FRT* chromosome after being clipped according to a preferred embodiment of the present invention.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0056] The present invention will now be described more specifically with reference to the following embodiments. ~~An advance way is provided to circumvent the previous problem by~~  
An advancement is provided which circumvents previous problems for generating a new  
"double *FRT*" chromosome (where the "double *FRT*" chromosome was originally generated by



Chou and Perrimon, 1996) which is insensitive to  $\Delta 2$ -3 transposase. On the double *FRT* chromosome, two *P[FRT]* insertions are located at the base of each chromosomal arm, as the targets for FLP recombinase. These original double *P[FRT]* insertions were specifically designed to facilitate the large scale recombination clonal analysis of autosomal genes. However, the *P* transposase target sequences [[12]] contained in *P[FRT]* transposon are sensitive and will respond to the presence of P-transposase in the genome designed for P transposon-directed mutagenesis. To modify the double FRT chromosomes so that they are no longer sensitive to P-transposase will be the first step toward the strategy of using the P transposon-directed, but not the EMS, mutagenesis in genome-wide screening.

[0057] P donor elements are frequently excised and reinserted locally (Zhang and Spradling, 1993, Genetics 133, 361). The local transposition derivatives can be the candidates for the imprecise transposition events among which the P transposase target sequences of *P[FRT]* have been impaired and become insensitive to the P-transposase activity. Experimentally, several steps need to be repeatedly operated.

[0058] 1. Double *FRT* chromosomes will be exposed to the P-transposase for local and imprecise transposition to occur.

[0059] 2. Both double *FRT* chromosomes contain one *P[FRT]* insertion with *mini-white*<sup>+</sup> gene on one arm and another *P[FRT]* with *rosy*<sup>+</sup> gene on the other arm, wherein both the *mini-white*<sup>+</sup> gene and the *rosy*<sup>+</sup> gene are selection marker genes. The imprecise transposition event will be followed by the mosaic white eyes in one arm or the loss of *rosy*<sup>+</sup> gene in another arm.

[0060] 3. The two *P[FRT]* insertions insensitive to P-transposase can be first screened ~~subject to~~ for an immobility of *miniwhite* marker. The mosaic white eyes will reflect the persistent transposition of *P[FRT]* and the non-mosaic white-plus eyes will represent the insensitivity to the P-transposase.

[0061] 4. The FRT derivatives with non-mosaic white-plus eyes will be the source for the search for the loss of *rosy*<sup>+</sup> gene in another arm. Derivatives ~~fulfilled with~~ meeting both criteria will be examined for both the recombination capability, which represents the functional activity of *P[FRT]* insertion and its homologous location in compared with original *P[FRT]* insertion, and the homozygous viability, which represents the genetic background which is not detectably changed after its exposure to the P transposase.

[0062] 5. The recombination accessibility of FRT sequences will be examined by the presence of FLP. Either germline or somatic recombination clone occurrence can be used to estimate the recombination event.

[0063] 6. The stabilized or clipped *P[FRT]* insertion candidates will be challenged by the P-transposase for the second time.

[0064] 7. The FRT sequences insensitive to P-transposase, the recombination accessibility of FRT and the homozygous viability will be examined again as described in steps 2-5.

[0065] 8. The steps 6 and 7 will be ~~cycled for~~ repeated at least twice to ensure the transposition insensitivity and recombination accessibility and homozygous viability of the double *FRT* chromosomes.

[0066] 9. The actual molecular nature of the clipped insertions can be examined by PCR using primer sets encompassing the target sequences of the P transposase.

[0067] After three rounds of P-transposase treatment, one derivative of the original  $FRT^{2L2R}$  chromosome was selected as the modified and advanced  $cFRT^{2L2R}$  chromosome which is insensitive to P-transposase challenges. However, the  $cFRT^{2L2R}$  chromosome is functional for ~~the occurring of the~~ recombination on both 2L and 2R arm when using the original homologous *P[FRT]* insertions for germline clonal analysis. This homozygous viable  $cFRT^{2L2R}$  chromosome is ~~absent of~~ lacking both P5' inverted repeat sequences required for P transposition at both 2L and 2R *P[FRT]* insertions. ~~These damages~~ This loss may ~~[[cause]]~~ leave this chromosome insensitive to P-transposase. In other words, the *Drosophila*  $cFRT^{2L2R}$  chromosome contains a  $cFRT^{2L}$  which is a clipped *P[FRT]* insertion on the left arm, and a  $cFRT^{2R}$  which is a clipped *P[FRT]* insertion on the right arm. The  $cFRT^{2L}$  and the  $cFRT^{2R}$  are insensitive to P transposase and can not be mobilized by P transposase.

[0068] In addition, in another embodiment of the present invention, based on the imprecise transposition and local transposition, the original double  $FRT^{2L2R}$  chromosome was challenged with serial rounds of  $\Delta 2-3$  transposase treatment. This chromosome contains the *P[FRT40A]* insertion carrying *rosy*<sup>+</sup> eyecolor marker and the *P[FRT42B]* insertion carrying *miniwhite*<sup>+</sup> eyecolor marker. Mosaic *miniwhite*<sup>+</sup> eyecolor production represents the responsiveness of the *P[FRT]* insertion to the P transposase. After the first two rounds of challenge, derivative chromosomes with the loss of both mosaic *miniwhite*<sup>+</sup> and *rosy*<sup>+</sup> eyecolor markers are selected for further treatment as long as they are homozygously viable and functional for germline clone production based on the FLP-DFS technique. Then, further rounds of treatment will select those

derivatives that produce offspring all ~~behaved~~ behaving similarly to parent chromosomes in terms of being homozygous viable with compatible recombination frequency. One candidate meeting the criteria has been shown to be ~~absent of~~ lacking P5' inverted repeat sequences required for *P* transposition in both 2L and 2R P[FRT] insertions.

[0069] This unique chromosome is deemed the "*cFRT<sup>2L2R</sup>*", which particularly combines the *P* transposon and the FLP-FRT site-specific recombination system as an integrated one for almost 40% of *Drosophila* genome. It allows the direct use of *P* transposon as mutagen for the ~~entry of~~ molecular cloning [~~drill~~] and simultaneously the functional analysis of the genes disrupted by *P* insertions. This is first examined by using the FLP-DFS technique for a small scale of germline clone (GLC) screen for 36 *P*-induced essential loci. Specific maternal effect phenotypes can be simultaneously characterized with respect to their GLC phenotypes and their molecular properties can be assigned according to the flanking genomic DNA sequences recovered by plasmid rescue and inverted PCR methods for the *P* insertions.

[0070] In order to describe the present invention in a greater detail, the material and method used in the embodiments are disclosed below. Flies used here were raised on standard *Drosophila* media at 25°C unless indicated. Description of balancers and mutations can be found in Lindsley and Zimm (1992). The jumpstarter stock strain utilized here is *Δ2-3(P[ry<sup>+</sup>;Δ2-3]99B)* that carries a defective P element on the third chromosome at 99B which constitutively express high levels of transposase but can not itself transpose (Robertson et al. 1988). The *yw; Δ2-3, Sb/TM6, Ubx* stock was obtained from the Bloomington Stock Center.

[0071] The autosomal FLP-DFS technique is used for the production of germ line mosaics. To generate homozygous GLCs, females were crossed with males of genotype *ywFLP<sup>12</sup>/Y; CyO/P[ovo<sup>D1</sup>]<sup>2L</sup>FRT<sup>2L</sup>* or *ywFLP<sup>12</sup>/Y; CyO/P[ovo<sup>D1</sup>]<sup>2R</sup>FRT<sup>2R</sup>*. These males were generated by crossing females from the *ywFLP<sup>12</sup>; CyO/Sco* with the appropriate *P[ovo<sup>D1</sup>]/FRT* males. Females of appropriate genotypes were allowed to lay eggs for 1 day in vials and the progeny heat shocked once for 2 hrs at 37°C in a circulating water bath over a period of 2 days when they reached late L2 to L3 larval stages as described (Chou and Perrimon, 1996). Subsequently, females of the appropriate genotype were analyzed for the presence of GLC ovaries.

[0072] Germline clones in females heterozygous for *ovo<sup>D1</sup>* were identified by ovary dissection. Females were dissected four days following eclosion. Since the *ovo<sup>D1</sup>* mutation perturbs early oogenesis, a germline clone is identified by the presence of vitellogenic egg

chambers. The percentage of mosaic ovaries (%MO) is calculated by dividing the number of ovaries carrying vitellogenic egg chambers with the total number of ovaries examined.

[0073] Chromosomes derived from  $FRT^{2L2R}$  were used as genomic DNA source for PCR examination. Single fly of various genotypes were used for the DNA isolation. PCR reaction was performed using touch-down melting temperature from 65-55°C. The presence of P5' and P3' regions were detected by standard PCR reaction. The primer sets used are listed below.

IR (1-20)	CATGATGAAATAACATAAGG (SEQ ID NO:1)
P5' sense (26-45)	CCGTCGAAAGCCGAAGCTTA (SEQ ID NO:2)
P5' antisense (264-232)	CCCAAGGCTCTGCTCCCACAATT (SEQ ID NO:3)
P3' sense (2756-2775)	AAACCCCACGGACATGCTAA (SEQ ID NO:4)
P3' antisense (2878-2839)	CGGCAAGAGACATCCACTTA (SEQ ID NO:5)

[0074] The information of the *P[lArB]* element insertions is examined by inverse PCR. A sample of genomic DNA equivalent to 5-6 flies was digested with restriction enzymes Pvu II, Sau 3AI, or HinP1I separately. The digested DNA was religated with T4 DNA ligase at low DNA concentration about 0.5 ng/μl DNA. The ligation mix was extracted with phenol-chloroform, precipitated with ethanol and finally dissolved in 20μl distilled water. 2μl of each product was used for PCR amplification. Plac1 and Splac2 primers (designed by BDGP) were used for first P insertion 5' end PCR amplification for 25 cycles. The amplified products were diluted 50-fold, and 1μl of the diluted product was used for a second round of PCR for 30 cycles using two nested primers, sp1 and Splac2 (designed by BDGP). Subsequently, the amplified product was sequenced directly using a nested primer, Sp1 or Splac2.

[0075] The information of the  $FRT^{2L}$  and  $FRT^{2R}$  insertions is examined by inverse PCR. All the reaction conditions are similar to the above description, except primers and restriction enzymes used. For  $FRT^{2L}$ , the Sau3A enzyme was used to digest genomic DNA, and Pry1 and Pry2 primers (designed by BDGP) were used for IPCR, and Sp3 (designed by BDGP) primer was used for DNA sequencing. For  $FRT^{2R}$ , the HipI enzyme was used to digest genomic DNA, and Pry1 and Pry2 primers (designed by BDGP) were used for IPCR, and Pry1 (designed by BDGP) primer was used for DNA sequencing. The primer sequences are listed below.

Plac1	CAC CCA AGG CTC TGC TCC CAC AAT (SEQ ID NO:6)
Pry1	CCT TAG CAT GTC CGT GGG GTT TGA AT (SEQ ID NO:7)
Pry2	CTT GCC GAC GGG ACC ACC TTA TGT TAT T (SEQ ID NO:8)
Splac2	GAA TTC ACT GGC CGT CGT TTT ACA A (SEQ ID NO:9)
Sp1	ACA CAA CCT TTC CTC TCA ACA A (SEQ ID NO:10)
Sp3	GAG TAC GCA AAG CTT TAA CTA TGT (SEQ ID NO:11)

[0076] ~~As to the respect of~~ For the plasmid rescue method, a sample of genomic DNA equivalent to 5-6 flies was digested with appropriate restriction enzymes separately (e.g. XbaI and Sac II for PZ line, Hind III and Kpn I for *P[lArB]* line), then ligated with T4 DNA ligase to circularize the restriction fragments. The ligation mix was precipitated with ethanol and dissolved in 2 µl distilled water. 1 µl of the ligation DNA was added to 20 µl of the competent cells (DH10α). The competent cells were transformed by electroporation. The resulting colonies were pick and plasmid was extracted by using mini-preparation method. The plasmid was cut by single cut (e.g. Hind III for Plar B line) and double cut (e.g. Hind III and Sac II for Plar B line) to check size in agarose gel. Finally, the colony with correct size plasmid was incubated and sequenced using primer pry2 (designed by BDGP).

[0077] ~~As to the respect of~~ For the method of Intrachromosomal flip-out experiment, the intrachromosomal flip-out experiment described previously (Golic and Linquist, 1989) is followed to removed miniwhite+ marker in *cFRT<sup>2L2R</sup>* chromosome. The 24-36 hours offspring from the *wY; cFRT<sup>2L2R</sup>/cFRT<sup>2L2R</sup>* males crossed with *ywFLP<sup>12</sup>; CyO/Sco* females were heat shock treated for 2 hrs at 37°C. The adult progeny were crossed with *w/w; CyO/Sco* females to examine the possible presence of white eyecolor offspring. The removal of miniwhite+ marker will produce white eyecolor progeny as the result of intrachromosomal FRT-FRT recombination.

[0078] As to the EMS mutagenesis method, 70-90 isogenized *cFRT<sup>2L2R</sup>* males were fed with a mixture of 5mM EMS and 1 % glucose solution for one day. These EMS-treated flies were mated to *w/w; Sco/CyO* females. White-eye male progenies which indicate that the loss-of-function of the *miniwhite* gene on the *cFRT<sup>2R</sup>* sequence were maintained for further analyses.

[0079] The DNA configuration of *clipped FRT* chromosome is determined by PCR. After IPCR and plasmid rescue manipulations, the *P[FRT40A]* and the *P[FRT42B]* insertion points were determined (the results are shown in the following embodiments). The molecular nature of

the clipped  $P[hsneo>>, ry^+, FRT]^{40A}$  and clipped  $P[>w^{hs}>, FRT]^{42B}$  of *hL18C1* were further defined by different primer sets to refine the configuration of the modified  $P[FRT]$  insertions.

[0080] The primers used are defined by the 5' to 3' sequence base pair numbered in the FBtp0000348 locus, that is the  $P[hsneo>>, ry^+, FRT]$  element, and AE003781 genomic clones for clipped  $P[hsneo>>, ry^+, FRT]^{40A}$ , and in the FBtp0000268 locus, that is the  $P[>w^{hs}>, FRT]$  element, and AE003789 genomic clones for *clipped*  $P[>w^{hs}>, FRT]^{42B}$ .

Primers used for *clipped*  $P[hsneo>>, ry^+, FRT]^{40A}$  PCR reactions (SEQ ID NOS: 12-25):

40SE	CGACGAGTTGCTTCTCCCACA	240516-240536 in AE003781 clone
40AS	GTTTCCCTCGCACTGCTATTT	240952-240932 in AE003781 clone
5'SE	CCGTCGAAAGCCGAAGCTTA	26-45 in FBtp0000348 locus
5'AS	CCCAAGGCTCTGCTCCCACAATT	254-232 in FBtp0000348 locus
ry1	CGCACGGTTTCAATCACA	948-930 in FBtp0000348 locus
ry2	GGTTACGAGGCAGCAGTTCTA	2070-2050 in FBtp0000348 locus
ry3	AACGCCCACTTCCGTATTGC	4035-4016 in FBtp0000348 locus
ry4	AATCCTGGTGCTTGCTTTCCT	6092-6072 in FBtp0000348 locus
Hsp	GTAGGTCATTTGTTTGGCA	8028-8046 in FBtp0000348 locus
neo	CTGATGCCGCCGTGTTC	8587-8603 in FBtp0000348 locus
FRTf	CCCCGCATGGAATGGGATAAT	9609-9629 and 10326-10346 in FBtp0000348 locus
FRT <sub>r</sub>	AGTCCGGTGCGTTTTT	9948-9933 and 10665-10650 in FBtp0000348 locus
3'SE	AAACCCCACGGACATGCTAA,	14861-14880 in FBtp0000348 locus
3'AS	CGGCAAGAGACATCCACTTA.	14993-14974 in FBtp0000348 locus

Primers used for *clipped*  $P[>w^{hs}>, FRT]^{42B}$  PCR reactions are listed below (SEQ ID NOS: 26-33)

42SE	TGCTCGCTTGGATGAAC	11032-11047 in AE003789 clone
42AS	AGTGGAGTGGGAGTGGA	11600-11584 in AE003789 clone
5'SE	CCGTCGAAAGCCGAAGCTTA	26-45 in FBtp0000268 locus
5'AS	CCCAAGGCTCTGCTCCCACAATT	254-232 in FBtp0000268 locus
FRTf	CCCCGCATGGAATGGGATAAT	2549-2529 and 7937-7917 in FBtp0000268 locus
FRT <sub>r</sub>	AGTCCGGTGCGTTTTT	2210-2225 and 7598-7613 in FBtp0000268 locus
3'SE	AAACCCCACGGACATGCTAA	15101-15120 in FBtp0000268 locus
3'AS	CGGCAAGAGACATCCACTTA	15214-15233 in FBtp0000268 locus

[0081] In order to describe the present invention in a greater detail, the experimental data are disclosed below. The  $FRT^{2L2R}$  chromosome contains  $P[hsneo>>, ry^+, FRT]$  insertion on 40A

and  $P[>w^{hs}>, FRT]$  insertion on 42B (Chou and Perrimon, 1996). The treatment of this double  $P[FRT]$  chromosome with  $\Delta 2-3$  transposase is expected to produce mosaic miniwhite<sup>+</sup> eyecolor after the first round treatment and to recover non-miniwhite-mosaic eye color phenotype after the second challenge if the  $P[>w^{hs}>, FRT]$  transposon is not sensitive to  $\Delta 2-3$  transposase (as shown below).

$\Delta 2-3$  transposase treatment for the recovery of the loss of mosaic miniwhite eyecolor production

1.  $w/Y; FRT^{2L2R} / FRT^{2L2R} Xyw/yw; \Delta 2-3, Sb/TM6, Ubx$
2.  $yw/Y; FRT^{2L2R} /+; \Delta 2-3, Sb/+ Xyw/yw; \Delta 2-3, Sb/TM6, Ubx$
3.  $yw/Y; (FRT^{2L2R})^c/+; \Delta 2-3, Sb/+ or TM6, Ubx X w/w; CyO/Sco$
4. Pick up males without mosaic miniwhite eyecolor and set up individual lines.

[0082] After two rounds of treatments, it is also expected that the loss of  $ry+$  eyecolor will represent the imprecise excision of  $P[hsneo>>, ry+, FRT]$  transposon (as shown below).

Selection of  $(FRT^{2L2R})^c$  chromosome with the loss of  $rosy+$  eyecolor

1.  $w/Y; (FRT^{2L2R})^c/(FRT^{2L2R})^c X +/BsY; Sp/CyO; MKRS/TM2,ry$
2.  $+/Y; (FRT^{2L2R})^c/CyO; MKRS/+ X ry/ry$
3.  $+/Y; (FRT^{2L2R})^c/+; MKRS,ry/ry$
4. search for  $(FRT^{2L2R})^c$  chromosome which is lost of  $ry+$  eyecolor

[0083] From 427 independent  $FRT^{2L2R}$  derived chromosomes which displayed nonmosaic miniwhite eyecolor, 107  $ry$  eyecolor lines are obtained. After GLC analysis of these lines, three lines are capable of performing recombination on both 2L and 2R arms and 6 lines on 2R arm only. Others displayed destroyed or very low frequency of recombination ability representing the mobilized  $P[FRT]$  producing a damaged FRT DNA sequences or, alternatively, a non-homologous condition with respect to  $P[FRT]ovo^{Dl}$  chromosome used.

[0084] The three lines,  $hL18$ ,  $hL92$ , and  $hL97$  are homozygously viable with comfortable ratio. Further treatments were focused on these three candidates.

[0085] Further treatments of chromosomes selected from previous challenges are examined by two criteria, homozygous viability and germ-line clone (GLC) recombination frequency. The lethality produced during transposase challenge would represent either the accumulation of mutations or the damage of loci flanking the previous P insertions caused by mobilization. GLC recombination will provide further information regarding the presence of FRT sequences, and possibly the degree of damage received.



[0086] The further treatments of the *hL18*, *hL92*, and *hL97* lines removed *hL92* as the possible candidate since 15 lines originated from *hL92* all displayed homozygous lethality. This clearly showed that *hL92* is very sensitive to the P transposase.

[0087] After the third round treatment, *hL97* derivatives showed weak sensitivity to the P transposase since different lines displayed fluctuated homozygous viability (as shown in Table 1A).

**Table 1A.**  
**The third round**

LINE	H.V.	%
<i>hL97C1</i>	2/34	6
<i>hL97C1'</i>	2/24	8
<i>hL97C3</i>	15/48	31
<i>hL97C3'</i>	10/30	33
<i>hL97C3''</i>	5/15	33
<i>hL97C4</i>	0/21	0
<i>hL97C4'</i>	0/33	0
<i>hL97C5</i>	13/60	17
<i>hL97C5'</i>	4/29	14
<i>hL97C6</i>	9/41	22
<i>hL97C14</i>	5/20	25
<i>hL97C16</i>	0/28	0
<i>hL97C16'</i>	0/30	0

HV: Homozygous viability is the number of homozygous flies divided by the number of total flies (homozygous + heterozygous). In theory, 33% would represent an expected homozygously viable rate.

GLC and %MO: Germ-line clonal frequency is calculated as the percentage of mosaic ovaries (%MO) of the females dissected. %MO=[number of ovaries with GLC clones/ total number of ovaries examined] x 100%.

[0088] *hL97C3''* line was treated further since homozygously viable stock can be established. After fourth round, homozygous viability displayed smaller degree of fluctuation compared with the result from the third round (as shown in Table 1B)

**Table 1B.**  
**The fourth round**

LINE	H.V.	%	2LGLC	% MO	2RGLC	%MO
<i>hL97C3''C1</i>	36/161	22	33/40	83		
<i>hL97C3''C3</i>	53/182	29				
<i>hL97C3''C4</i>	46/158	29				



LINE	H.V.	%	2LGLC	% MO	2RGLC	%MO
<i>hL97C3''C7</i>	139/446	31	129/194	67	87/100	87
<i>hL97C3''C8</i>	29/105	28				
<i>hL97C3''C10</i>	106/316	34				
<i>hL97C3''C12</i>	103/445	23	26/30	87	14/14	100
<i>hL97C3''C14</i>	12/90	8	54/84	64	41/48	85
<i>hL97C3''C15</i>	130/420	31	25/46	54	26/36	72
<i>hL97C3''C16</i>	6/45	13				
<i>hL97C3''C18</i>	18/111	16				
<i>hL97C3''C20</i>	70/351	20	40/64	63	71/98	72
<i>hL97C3''C21</i>	47/326	14				

[0089] However, only 8 out of 13 lines can be established as homozygously viable stocks. The result obtained from fifth round of treatment (as shown in Table 1C) suggests that few *hL97C3''* derivatives can tolerate the transposase challenges.

**Table 1C.**  
**The fifth round**

LINE	H.V.	%	2LGLC	% MO	2RGLC	%MO
<i>hL97C3''C1C1</i>	39/174	22	50/78	64	17/26	65
<i>hL97C3''C3C1</i>	19/93	20	38/66	58	22/30	73
<i>hL97C3''C3C2</i>	25/107	23	19/28	68	16/20	80
<i>hL97C3''C7C2</i>	18/48	38				
<i>hL97C3''C7C3</i>	36/164	28	26/38	68	46/58	79
<i>hL97C3''C7C5</i>	13/48	27				
<i>hL97C3''C15C1</i>	40/81	49				

[0090] After the third round of transposase treatment, 2 independent derivatives, *hL18c1* and *hL18c3* were picked up for further treatment since both are homozygously viable and have 55 to 77% recombination frequency on both 2L and 2R arms (data not shown). After the fourth round, 11 derivatives from *hL18c1* all display expected homozygous viability ratio and constant recombination frequency ranging from 55 to 80 (as shown in Table 2A).

**Table 2A**  
***hL18C1***

LINE	H.V.	%	2LGLC	% MO	2RGLC	%MO
<i>hL18c1c1</i>	95/280	32	43/64	67	41/58	71
<i>hL18c1c2</i>	88/248	35	35/56	63	38/56	68
<i>hL18c1c3</i>	90/229	39	45/68	66	26/34	76
<i>hL18c1c4</i>	107/231	46	83/98	85	40/52	77

LINE	H.V.	%	2LGLC	% MO	2RGLC	%MO
<i>hL18c1c5</i>	89/231	38	66/82	80	28/50	56
<i>hL18c1c6</i>	97/232	42	76/110	69	30/54	56
<i>hL18c1c7</i>	79/226	35	108/182	59	50/66	76
<i>hL18c1c8</i>	82/232	35	57/90	63	25/42	60
<i>hL18c1c9</i>	116/282	41	74/118	63	5/12	42
<i>hL18c1c10</i>	89/251	35	60/90	67	30/40	75
<i>hL18c1c11</i>	104/302	34	43/68	63	29/52	56

[0091] These results meet the criteria as the *hL18c1* chromosome is devoid of transposase sensitivity. Nevertheless, it is still able to perform the FLP-FRT recombination with a comfortable frequency.

[0092] 1 out of 11 *hL18C3* derived lines showed homozygous lethality. In comparison with *hL18c1*, this suggests that the *hL18c3* chromosome is remaining sensitive to the P transposase in a low frequency manner (as shown in Table 2B).

**Table 2B.**  
**hL18C3**

LINE	H.V.	%	2LGLC	% MO	2RGLC	%MO
<i>hL18c3c1</i>	76/265	29	71/94	76	51/74	69
<i>hL18c3c2</i>	120/301	40	51/82	62	26/58	45
<i>hL18c3c3</i>	83/218	38	77/110	70	32/56	57
<i>hL18c3c4</i>	101/255	40	139/196	41	49/56	88
<i>hL18c3c5</i>	0/120	0				
<i>hL18c3c6</i>	144/293	49	27/58	47	37/62	60
<i>hL18c3c7</i>	97/262	37	41/74	55	22/50	44
<i>hL18c3c8</i>	75/231	32	29/48	60	22/40	55
<i>hL18c3c9</i>	95/298	32	30/58	52	30/50	60
<i>hL18c3c10</i>	85/245	35	60/82	73	8/16	50
<i>hL18c3c11</i>	101/240	42	23/56	41	41/64	64

[0093] The molecular nature of *hL18C1* was further defined first by IPCR to define the loci of  $P[hsneo>>, ry^+, FRT]^{40A}$  and  $P[>w^{hs}>, FRT]^{42B}$ , and second by different primer sets to refine the configuration of the modified  $P[FRT]$  insertions.

[0094] Please refer to Fig. 1, which is a diagram showing the position of the  $P[FRT]$  insertion on the left arm of the *FRT* chromosome before being clipped according to a preferred embodiment of the present invention. Originally,  $P[hsneo>>, ry^+, FRT]^{40A}$  is inserted into the 3' end to the base T at 240696 bp of the AE003781 clone with the P3' end facing centromere. Please refer to Fig. 2, which is a diagram showing the  $P[FRT]$  insertion on the left arm of the

*FRT* chromosome before being clipped according to a preferred embodiment of the present invention. The *P[FRT]* insertion on the left arm includes P5' region (1-157 bp, green color), pUC8 (547-704 bp and 10892-13391 bp, white color), *rosy* (922-3164 bp and 3171-7992 bp, red color), Hsp70Bb (8003-8449 bp and 13412-13858 bp, purple color), neoR (8463-9457 bp and 13872-14866 bp, blue color), FRT (9468-10174 bp and 10185-10891 bp, yellow color), and P3' region (15041-15262 bp, pink color). Please refer to Fig. 3, which is a diagram showing the *P[FRT]* insertion on the left arm of the *FRT* chromosome after being clipped according to a preferred embodiment of the present invention. In clipped *P[hsneo>>, ry+, FRT]40A*, imprecise excision caused the removal of P5' region and most part of the *rosy+* DNA segment, e.g. fragment from bases 26 to around 2070 of FBtp0000348 locus were known being deleted.

[0095] Please refer to Fig. 4, which is a diagram showing the position of the *P[FRT]* insertion on the right arm of the *FRT* chromosome before being clipped according to a preferred embodiment of the present invention. *P[>whs>, FRT]42B* is originally inserted into the 3' end to the base T at 11497 bp of the AE003789 clone with the P5' end pointing toward telomere. Please refer to Fig. 5, which is a diagram showing the *P[FRT]* insertion on the right arm of the *FRT* chromosome before being clipped according to a preferred embodiment of the present invention. The *P[FRT]* insertion on the right arm includes P5' region (1-587 bp, green color), FRT (594-2690 bp and 7372-8078 bp, yellow color), mini-white (2728-2937 bp, 2944-6413 bp and 6434-6966 bp, white color), Hsp70A (6981-7349 bp, purple color), and P3' region (15041-15262 bp, pink color). Please refer to Fig. 6, which is a diagram showing the *P[FRT]* insertion on the right arm of the *FRT* chromosome after being clipped according to a preferred embodiment of the present invention. Most of the most P5' region and one of the FRT DNA repeats, e.g. bases 10 to 2821 of FBtp0000268 locus, were deleted in the clipped *P[>whs>, FRT]42B* during this trimming action. The clipped *P[FRT]* insertion on the right arm includes P5' region (1-9 bp, green color), FRT (4560-5266 bp, yellow color), mini-white (10-125 bp, 132-3601 bp and 3622-4154 bp, white color), Hsp70A (4169-4537 bp, purple color), and P3' region (5297-5529 bp, pink color).

[0096] The P transposase recognizes the 5' and 3' ends including inverted repeat and other internal sequences as targets of transposase for *P* transposition (Kaufman et al., 1989). The configuration suggests that both clipped *P[hsneo>>, ry<sup>+</sup>, FRT]<sup>40A</sup>* and clipped *P[>w<sup>hs</sup>>, FRT]<sup>42B</sup>* in this new chromosome are lacking of the P5' region. Nevertheless, FRT DNA sequences remain and are still fully functional for FRT-based site-specific recombination. Due

to the clipping away of the two *P5'* substrate regions, *hL18c1* are renamed the "*clipped FRT<sup>2L2R</sup>* (*cFRT<sup>2L2R</sup>*)" chromosome for the *clipped P[FRT]<sup>2L</sup>* *clipped P[FRT]<sup>2R</sup>* transposon insertions on the second chromosome.

[0097] *P[>w<sup>hs</sup>>, FRT]* insertion on 42B provides the miniwhite marker of *FRT<sup>2L2R</sup>* chromosome. The mini white marker can be removed by intrachromosomal recombination between the two FRT DNA sequences (Golic and Lindquist, 1989). To perform *P*-directed mutagenesis scheme using *P[lacW]* instead of *P[ry+]* transposon, the miniwhite marker from *cFRT<sup>2L2R</sup>* chromosome was attempted to be removed. In contrast to positive result when *hL18* is treated, the miniwhite marker cannot be removed from *cFRT* chromosome. At least five trials were done by different people showing the same result. Though the clipped *P[>w<sup>hs</sup>>, FRT]* insertion on 42B can accept the *P[>w<sup>hs</sup>>, FRT]* insertion on the homologous chromosome for interchromosomal recombination, it can not perform the intrachromosomal recombination.

[0098] To search for *cFRT<sup>2L2R</sup>* chromosome with *white-minus* eyecolor, about 18,000 flies were examined after EMS treatment of the isogenized *IcFRT<sup>2L2R</sup>*#60 (as shown in Table 3). 4 flies, EMS 1 to 4, were selected.

**Table 3.**  
**Germline clonal analysis of isogenized *cFRT<sup>2L2R</sup>* chromosome**

	MO%		Hatching rate in GLC		DE%	
<i>IcFRT<sup>#60</sup></i> -2L	156/184	0.85%	493/595	0.83%	5/595	0.0084%
<i>IcFRT<sup>#60</sup></i> -2R	153/178	0.86%	436/450	0.97%	3/450	0.0067%
<i>IcFRT<sup>#61</sup></i> -2L	148/162	0.91%	563/600	0.94%	6/600	0.0100%
<i>IcFRT<sup>#61</sup></i> -2R	132/158	0.84%	417/430	0.97%	3/430	0.0070%

MO %: mosaic ovary (NO. of developed ovary versus total ovary).

Hatch rate in GLC: NO. of hatched eggs/total eggs laid.

DE %: NO. of dead embryos/total eggs in germline clone analysis

[0099] EMS 1 had cream-colored eyes and the others with white and red mosaic eyecolors. After these 4 males were crossed with *w/w; Sco/CyO* females, different results for these 4 chromosomes were found. All the progeny of EMS 1 had red eyes. EMS3 turned out to be male sterile. The progeny of EMS2 and EMS4 had either red eyes or white eyes with the ratio of 1:1. One red-eyed male and 5 white-eyed males from the EMS2 progeny and one white-eyed male from the EMS4 progeny were collected for further analyses. These putative *cFRT<sup>2R2LW</sup>* chromosomes were denominated NR2-1, NW2-1 to NW2-5 and NW4, respectively.

[0100] To examine the recombination capacities of these modified chromosomes, germline clone analysis is generated. It turned out that the hatching rate of each strain was similar to the original untreated strain  $IcFRT^{2L2R}$ #60 (as shown in Table 4), and no particular embryonic phenotype was found.

**Table 4**  
**Hatching rate in GLC of  $cFRT^{2L2RW}$  chromosomes**

	GLC tested	Hatching rate in GLC
$IcFRT^{60}$	2L	493/595=82.9%
	2R	436/450=96.9%
NR2-1	2L	243/268=90.7%
	2R	208/227=91.6%
NW2-1	2L	178/200=89.0%
	2R	160/192=83.3%
NW2-2	2L	168/189=88.9%
	2R	160/175=91.4%
NW2-3	2L	120/141=85.1%
	2R	215/227=94.7%
NW2-4	2L	159/179=88.8%
	2R	159/187=85.0%
NW2-5	2L	131/155=84.5%
NW4	2L	211/219=96.3%
	2R	173/185=93.5%

[0101] After isogenization, it was confirmed that the chromosome behaved as a wild type chromosome (as shown in Table 3). This  $cFRT^{2L2R}$  chromosome was used for a pioneer screen for the searching of essential loci on the second chromosome with specific maternal effects.

[0102] The failure to remove the miniwhite marker enforced us to use  $P[ry+]$  transposon as the mutagen. Two schemes described below were designed.

**A.**

1.  $+/Y; SM1/Sco; ry/ry \times P[lArB]/P[lArB]$
2.  $P[lArB]/Y; SM1/+; ry/+ \times w/w; cFRT/cFRT$
3.  $yw/Y; Sco/CyO, \Delta 2-3; MKRS/+ \times P[lArB]/w; cFRT/SM1; +/+$
4.  $P[lArB]/Y; cFRT/CyO, \Delta 2-3; MKRS/+ \times SM1/Sco; ry/ry$
5. look for  $(+/Y; cFRT/SM1 \text{ or } Sco; MKRS/ry)$  males with  $ry+$  eyecolor
6.  $(+/Y; cFRT/SM1 \text{ or } Sco; MKRS/ry) \times SM1/Sco; ry/ry$ 
  - a. if all  $Sb$  flies are with  $ry^+$  eyecolor, the P transposon may be on the MKRS chromosome

- b. if some *Sb* and some *Sb*<sup>+</sup> flies are with *ry*<sup>+</sup> eyecolor, the P transposon may be on the cFRT or the fourth chromosome.
  - c. if all *Sb* and all *Sb*<sup>+</sup> flies are with *ry*<sup>+</sup> eyecolor, there may be multiple insertions on autosomes.
7. Select P transposon on cFRT chromosome and determine their homozygous lethality.

**B.**

1. *P[lArB]/P[lArB]; cFRT/cFRT; ry/ry X yw/Y; Sco/CyO, Δ2-3; MKRS/+*
2. *P[lArB]/Y; cFRT<sup>2L2R</sup>/CyO, Δ2-3; MKRS/+ X +/+; Sco/CyO; ry/ry*
3. Look for *(+/Y; cFRT<sup>2L2R</sup> / CyO or Sco; MKRS/ry)* males with *ry*<sup>+</sup> eyecolor
4. *(+/Y; cFRT/SM1 or Sco; MKRS/ry)* with *ry*<sup>+</sup> eyecolor X *+/+; Sco/CyO; ry/ry*
5. examine the location of *P[lArB]* insertions as that described in A. scheme.

[0103] Several different *P[lArB]* insertions on X chromosome were tested for their transposition efficiency. *P[lArB]* insertions with normal efficiency (Spradling et al., 1999) of transposition will be candidates for future large scale screening. The *P* transposon on X chromosome was used as the mutagen, determined whether the *P*-induced mutations on the second chromosome were created by the disruption of essential genes causing the homozygous lethality, analyzed the possible maternal functions based on the FLP-DFS technique (Chou and Perrimon, 1996), and recovered genomic DNA segments flanking *P* insertions by plasmid rescue and inversed PCR methods.

[0104] From 189 lines with *P* insertions on the *cFRT<sup>2L2R</sup>* chromosome, 36 homozygous lethal lines are obtained. The 19 % homozygous lethal rate is compatible with previous *P*-induced homozygous rate (Spradling et al., 1999). This further demonstrated that the *cFRT<sup>2L2R</sup>* can perform normal as a wild type chromosome for *P* transposition. As shown in Table 5, the four classes of zygotic lethals with specific maternal effect phenotypes (Perrimon et al., 1989): germ cell lethal (GCL) phenotype, abnormal oogenesis (AO) phenotype, maternal effect (ME) and maternal effect rescuable (MER) and no maternal effect (NME) phenotype can be assigned to the essential loci on the *cFRT<sup>2L2R</sup>* chromosome.

**Table 5**  
**Result of the primary screen for essential loci with specific maternal effects using the *cFRT2L2R* chromosome**

Stock #	2L	2R	Phenotype	Gene	Function	Allele
<b>Germ Cell Lethal phenotype</b>						
<i>1168c2</i>	<u>no eggs</u>	NME	arrested at stage 2	<i>CG13790</i>		New
				<i>CG13791</i>		New
<i>a150a</i>	<u>no eggs</u>	NME	arrested at stage 2	<i>AC3</i>	Guanylate cyclases	New
				<i>CG1512</i>	cell cycle regulator (cullin)	New
<i>a138</i>	<u>no eggs</u>		arrested at stage 2	<i>CycE</i>	cell cycle regulator (cullin)	Known
<i>R069</i>	<u>no eggs</u>	NME	arrested at stage 2	<i>CG9586/</i>		Known
				<i>Cg13108</i>		New
<i>1168-2</i>	<u>no eggs</u>	NME	arrested at stage 2			ND
<i>a137</i>	<u>NME</u>	<u>no eggs</u>	arrested at stage 2			ND
<i>1168e</i>	<u>NME</u>	<u>no eggs</u>	arrested at stage 2			ND
<b>Abnormal Oogenesis phenotype</b>						
<i>1164f2</i>	<u>AO</u>	NME	arrested at stage 6	<i>BcDNA:</i>	translation factor	New
				<i>GM14618</i>		
				<i>CG14040</i>	transporter	New
<i>b26b</i>	NME	<u>AO</u>	arrested at stage 6	<i>Sin3A</i>	transcription factor	Known
<i>b235</i>	NME	<u>AO</u>	arrested at stage 6			ND
<i>F263</i>	NME	<u>AO</u>	arrested at stage 6	<i>beta-tub56D</i>		New
<i>F284</i>	NME	<u>AO</u>	arrested at stage 6	<i>CG11508</i>	snRNA	New
<i>594-28</i>	NME	<u>AO</u>	arrested at stage 7	<i>CG12864</i>	motor protein	New
<b>Embryo with specific Maternal Effect phenotype</b>						
<i>Y016-1</i>	NME	<u>MER</u>	posterior group	<i>CG17280/2R</i>	Cytochrome C subunit	New
				<i>Or45b-</i>		
<i>b54a</i>	NME	<u>MER</u>	posterior group	<i>CG12931</i>	Olfactory receptor	New
				<i>CG1888</i>	NLS BP enzyme (RNA Pol 1)	New
<i>b347</i>	<u>MER</u>	NME	denticle belts fusion	<i>CG10685</i>		New
<i>b241b</i>	<u>ME</u>	NME	dorsalized denticle belts	<i>dorsal</i>	transcription factor	Known
<i>a210a</i>	<u>MER</u>	NME	Fusion	<i>Out at first</i>		Known
				<i>brat</i>		
<i>4799</i>	NME	<u>MER</u>	like gap gene lawn-of-denticles	<i>CG10719</i>	transcription factor	Known
			denticle belts	<i>tout-velu</i>	transmembrane protein	Known
<i>b345</i>	<u>ME</u>	NME	fusion (like kismet)			ND
<i>a350</i>	NME	MER	no phenotype			ND

Stock #	2L	2R	Phenotype	Gene	Function	Allele
<b>No maternal effect phenotype</b>						
<i>a22</i>	NME			<i>CG12052</i>	transcription factor	New
<i>b181</i>	NME	NME	denticle fusion	<i>Kismet</i>		Known
<i>1168f1</i>	NME	NME				ND
<i>1164b</i>	NME	NME				ND
<i>594c1</i>	NME	NME				ND
<i>1164</i>	NME	NME				ND
<i>1164c4</i>	NME	NME				ND
<i>1164a13</i>	NME	NME				ND
<i>a194</i>	NME	NME				ND
<i>a256</i>	NME	NME				ND
<i>a172b</i>	NME	NME				ND
<i>a301</i>	NME	NME				ND
<i>b187</i>	NME	NME				ND
<i>b140b</i>	NME	NME				ND
<i>b30a</i>	NME	NME				ND

Germ cell lethal phenotype is assigned if no eggs is laid for more than 15 GLC females examined.

AO: abnormal oogenesis; MER: paternal rescuable maternal effect; ME: strict maternal effect  
NME: no maternal effect; ND: molecular properties not yet determined. When there is no known alleles in FlyBase databank, the P insertion is said to be assigned as a new allele.

[0105] Either left or right arms can be the target of the *P* transposition and be analyzed for their germline clone phenotype. This is the first time to demonstrate that a double *FRT* chromosome can be used for GLC analysis for both arms simultaneously after *P* mutagenesis.

[0106] The molecular nature of these essential loci were immediately determined. From mutation lines analyzed, both new and known alleles were recovered. Known genes with specific GLC embryonic phenotypes were recovered. By complementation test and DNA sequences comparison, known genes with compatible phenotypical descriptions, for example, *dorsal* (*b241b*), *tout-velu* (4799) and *kismet* (*b181*), were confirmed both molecularly and genetically. These cases demonstrated that the mutation phenotypes obtained were resulted from the disruption of essential loci by *P* insertions instead of the presentation of unknown endogenous chromosomal denaturation created during the process of establishing the cFRT<sup>2L2R</sup> chromosome.

[0107] This preliminary result clearly demonstrates that the cFRT<sup>2L2R</sup> chromosome will be feasible for ~~[[the a]]~~ large scale screen using the *P*-transposon as the mutagen to disrupt 95% essential loci on the *Drosophila* second chromosome.



**[0108]** While the invention has been described in terms of what is presently considered to be the most practical and preferred embodiments, it is to be understood that the invention needs not be limited to the disclosed embodiments. On the contrary, it is intended to cover various modifications and similar arrangements included within the spirit and scope of the appended claims which are to be accorded with the broadest interpretation so as to encompass all such modifications and similar structures.

WHAT IS CLAIMED IS

1. A method for generating a *Drosophila* clipped *FRT* (cFRT) chromosome insensitive to a *P* transposase but remaining sensitive to a yeast site-specific flippase recombinase (FLP), comprising steps of:

(a) exposing a *FRT* chromosome to said *P* transposase for occurring a local and imprecise transposition, wherein said *FRT* chromosome contains a *P[FRT]* insertion with a selection marker gene;

(b) screening said *P[FRT]* insertion insensitive to said *P* transposase to obtain screened products;

(c) selecting candidate products from said screened products by further examinations; and

(d) exposing said candidate products by said *P* transposase and selecting a desired product by said further examinations to obtain said *Drosophila* clipped *FRT* (cFRT) chromosome is insensitive to said *P* transposase but remaining functional to yeast site-specific flippase recombinase.

2. The method according to claim 1, wherein said method further comprises a step (e) of examining the actual molecular nature of said clipped insertion by PCR (polymerase chain reaction).

3. The method according to claim 1, wherein said step (c) further comprises steps of:

(c1) examining said screened products for both recombination capability and homozygous viability; and

(c2) examining recombination accessibility of *FRT* sequences contained in a clipped *P[FRT]* insertion by the presence of said FLP to obtain said candidate products.

4. The method according to claim 3, wherein said recombination capability represents the functional activity of said clipped *P[FRT]* insertion and its homologous location relative to that of said original *P[FRT]* insertion.

5. The method according to claim 3, wherein said homozygous viability represents a genetic background after said chromosome's exposure to said *P* transposase.

6. The method according to claim 1, wherein said step (d) of exposing said candidate products by said *P* transposase and selecting said desired product by said further examinations is repeated at least twice.

7. The method according to claim 1, wherein said *Drosophila* cFRT chromosome is an isogenized homozygous viable *Drosophila* second chromosome.

8. The method according to claim 1, wherein said cFRT is formed due to a target sequence, recognized by said *P* transposase and responsible for a *P* transposase transposition, which is damaged and alternated into a type of incomplete target sequence, through one of a group consisting of:

- (1) missing of a P5' DNA sequence region;
- (2) missing of a P3' DNA sequence region; and
- (3) missing of DNA sequences other than those defined in item (1) and in item (2).

9. The method according to claim 1, wherein said *Drosophila* cFRT chromosome remains the functional activity of said cFRT insertion for a site-specific recombination in the presence of said FLP.

10. The method according to claim 1, wherein an effectiveness of said *Drosophila* cFRT chromosome is monitored by a FLP-FRT system and derived modification systems thereof.

11. The method according to claim 1, wherein an effectiveness of said cFRT chromosome is monitored by molecular biology methods for the description of said cFRT DNA sequences configuration.

12. The method according to claim 1, wherein said *Drosophila* cFRT chromosome remains to behave normally as a wild type chromosome feasible for various genetic manipulations.

13. The method according to claim 1, wherein a clipped *P[FRT]* insertion is alternatively moved to another chromosome from said *Drosophila* clipped I (cFRT) chromosome by treating said *Drosophila* cFRT chromosome with one of mutagens and X-ray.

14. The method according to claim 1, wherein said *Drosophila* cFRT chromosome is alternatively used to establish a *Drosophila* cell line based on a genetic background of said *Drosophila* cFRT chromosome.

15. The method according to claim 1, wherein said *Drosophila* cFRT chromosome is mutated to obtain gene mutations for further experiment.

16. The method according to claim 15, wherein a molecular information of said gene mutations is recovered by retrieving flanking DNA sequences of a clipped *P[FRT]* insertion with a molecular biology method.

17. The method according to claim 16, wherein said molecular biology method includes a plasmid rescue method, a inversed PCR method and a chromosomal walking method.

18. The method according to claim 16, wherein said molecular information of said gene mutations can be recovered by a related bioinformatic manipulation.

19. The method according to claim 18, wherein said related bioinformatic manipulation includes blasting databank, searching gene homologues of biological organisms, analyzing comparative genomics, and analyzing phylogenic distance and relationship.

20. The method according to claim 15, wherein the functional description of said gene mutations are further analyzed based on the information obtained from said molecular biology method and said related bioinformatic manipulation by using a biological technique.

21. The method according to claim 1, wherein said *Drosophila* cFRT chromosome is used to study the *Drosophila* genes located on the second chromosome and their corresponding gene homologues of other biological organisms including vertebrates, invertebrates, eukaryotes and prokaryotes.

22. A method for generating a *Drosophila* clipped  $FRT^{2L2R}$  ( $cFRT^{2L2R}$ ) chromosome insensitive to a *P* transposase but remaining functional to a yeast site-specific flippase recombinase (FLP), comprising steps of:

(a) exposing a double-FRY chromosome to said *P* transposase for occurring a local and imprecise transposition, wherein said double-*FRT* chromosome contains a first *P*[*FRT*] insertion with a first selection marker gene on one arm thereof and a second *P*[*FRT*] insertion with a second selection marker gene on the other arm thereof;

(b) screening respectively said first *P*[*FRT*] insertion and said second *P*[*FRT*] insertion insensitive to said *P* transposase to obtain screened products;

(c) selecting candidate products from said screened products by further examinations; and

(d) exposing said candidate products by said *P* transposase and selecting a desired product by said further examinations to obtain said *Drosophila* clipped  $FRT^{2L2R}$  ( $cFRT^{2L2R}$ ) chromosome insensitive to said *P* transposase but remaining functional to yeast site-specific flippase recombinase.

23. The method according to claim 22, wherein said method further comprises a step (e) of examining the actual molecular nature of said clipped insertions by PCR.

24. The method according to claim 22, wherein said step (b) further comprises steps of:

(b1) screening said first *P*[*FRT*] insertion insensitive to said *P* transposase subject to an immobility of said first selection marker gene; and

(b2) screening said second *P*[*FRT*] insertion insensitive to said *P* transposase from said screened products of step (b1) subject to an immobility of said second selection marker gene.

25. The method according to claim 22, wherein said step (b) further comprises steps of:

(b1') screening said second *P*[*FRT*] insertion insensitive to said *P* transposase subject to an immobility of said second selection marker gene; and

(b2') screening said first *P*[*FRT*] insertion insensitive to said *P* transposase from screened products of step (b1') subject to an immobility of said first selection marker gene.

26. The method according to claim 22, wherein said step (c) further comprises steps of:

(c1) examining said screened products for both recombination capability and homozygous viability; and

(c2) examining recombination accessibility of FRT sequences contained in said *P[FRT]* insertion by the presence of said FLP to obtain said candidate products.

27. The method according to claim 22, wherein said first selection marker is different from said second selection marker.

28. The method according to claim 22, wherein said *Drosophila* clipped  $FRT^{2L2R}$  chromosome is alternatively generated from two *Drosophila* clipped *FRT* (cFRT) chromosomes ( $cFRT^{2L}$  and  $cFRT^{2R}$  chromosomes) by a genetic recombination method.

29. A *Drosophila* clipped *FRT* (cFRT) chromosome, wherein said chromosome is insensitive to a P transposase but remains functional to a yeast site-specific flippase recombinase (FLP), comprising:

a *Drosophila* second chromosome main body; and

a clipped *P[FRT]* (cFRT) insertion immobilized by said P transposase.

30. The *Drosophila* cFRT chromosome according to claim 29, wherein said cFRT is formed due to a target sequence, recognized by said P transposase and responsible for a P transposase transposition, which is damaged and alternated into a type of incomplete target sequence, through one of a group consisting of:

(1) missing of a P5' DNA sequence region;

(2) missing of a P3' DNA sequence region; and

(3) missing of DNA sequences other than those defined in item (1) and in item (2).

31. A *Drosophila* clipped  $FRT^{2L2R}$  (cFRT<sup>2L2R</sup>) chromosome, wherein said chromosome is insensitive to a P transposase but remains functional to a yeast site-specific flippase recombinase (FLP), comprising:

a *Drosophila* second chromosome main body; and

a clipped *P[FRT]* (cFRT) insertion on a right arm (cFRT<sup>2R</sup>) of said *Drosophila* second chromosome and a clipped *P[FRT]* (cFRT) insertion on a left arm (cFRT<sup>2L</sup>) of said *Drosophila* second chromosome, wherein both said cFRT<sup>2R</sup> and said cFRT<sup>2L</sup> are immobilized by said *P* transposase.

32. The *Drosophila* cFRT<sup>2L2R</sup> chromosome according to claim 31, wherein said *P[FRT]* insertions on a left arm is inserted into the 3' end of the base T at 240696 bp of the AE003781 clone with the P3' end facing the centromere before being clipped, and said *P[FRT]* insertion a right arm is inserted into the 3' end of the base T at 11497 bp of the AE003789 clone with the P5' end pointing toward the telomere before being clipped.

33. The *Drosophila* cFRT<sup>2L2R</sup> chromosome according to claim 31, wherein said cFRT<sup>2L</sup> is an imprecise excision caused by a removal of P5' region and most part of a selection marker gene thereon, wherein a fragment from bases 26 to around 2070 of FBtp0000348 locus is deleted.

34. The *Drosophila* cFRT<sup>2L2R</sup> chromosome according to claim 31, wherein said cFRT<sup>2R</sup> is an imprecise excision caused by a removal of most of the P5' region and one of the FRT DNA repeats, wherein a fragment from bases 10 to 2821 of FBtp0000268 locus is deleted.

*DROSOPHILA* CLIPPED *FRT* (cFRT) CHROMOSOME  
INSENSITIVE TO *P* TRANSPOSASE, GENERATING  
METHOD THEREOF, AND APPLICATION THEREOF

ABSTRACT OF THE DISCLOSURE

A method for generating a *Drosophila* clipped *FRT* (cFRT) chromosome is provided, wherein the chromosome is ~~insensitive to~~ incapable of reacting with a *P* transposase but remains ~~functional to~~ capable of reacting with a yeast site-specific flippase recombinase (FLP). The method includes steps of: (a) exposing a *FRT* chromosome to the *P* transposase ~~for occurring~~ causing a local and imprecise transposition, wherein the *FRT* chromosome contains a *P[FRT]* insertion with a selection marker gene, (b) screening the *P[FRT]* insertion insensitive to the *P* transposase to obtain screened products, (c) selecting candidate products from the screened products by further examinations, and (d) exposing the candidate products by the *P* transposase and selecting a desired product by the further examinations to obtain the *Drosophila* clipped *FRT* (cFRT) chromosome ~~insensitive to~~ incapable of reacting with the *P* transposase but remaining ~~functional to~~ capable of reacting with the yeast site-specific flippase recombinase. The cFRT<sup>2L2R</sup> chromosome can be used as the direct target in the direct *P*-transposon-induced mutagenesis.